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**Developing Treatment, Treatment Validation, and Treatment Scope in the Setting of an Autism Clinical Trial**

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**14. ABSTRACT**

Initial IRB approval caused large and significant delays to this project. Unanticipated reduced recruitment rates also occurred. The SOW was changed accordingly. A restructuring along with changing responsibilities for some was done to help increase recruitment. This includes opening a second site for recruitment and treatment of subjects, in Newark NJ under Dr. X. Ming. This project has ended for initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0730 but partnering project W81XWH-08-1-0729 received and additional no-cost extension to part to increase the number of subjects completing this project. While a large number of inquiries were made about this study from our various recruiting sources. Many families decided to not join the study for several reasons. We obtained consent/assent from 49 subjects and assented 2. All of these 51 subjects underwent initial phenotype testing including ADOS, ADI, Vineland and Leiter. Fourty four of these subjects underwent medical examination. One subject was removed after initial blood chemistry and lab tests. Fourty three subjects were randomized and received either DHA, 200mg daily, or placebo, 11 of which dropped out. Thirty two of these subjects completed each of the study scales and checklists at each time point and completed the study. We are currently in the process of analyzing our data. We will finish analysis as soon as possible. If we are able to find additional funding we will modify and re-open this project to recruit new subjects to increase our statistical power. We plan on following up any positive data with grant applications to extend our findings as well as those of Partnering project W81XWH-08-1-0729 and Initiating project W81XWH-08-1-0728.

Please see initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0729.

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**Introduction:**

This project is to test to see if DHA treatment can beneficially affect excretion of urinary biomarkers of oxidative stress and the autism clinical phenotype. In addition polymorphic variants of genes of certain enzymes that synthesize and metabolize docosahexaenoic acid (DHA) may contribute to the phenotype of some autism cases. We will test to see if any of these genes are risk factors for autism. We will also measure changes in excretion of the polyunsaturated fatty acid (PUFA) derived biomarkers of oxidative stress (isoprostanes and neuroprostanes) together with the changes in production of anti-inflammatory lipid mediators. We will test these biomarkers to see if we can monitor and validate effectiveness of DHA therapy. We will also test the genotypes of key DHA-metabolizing enzymes can predict which patients will respond to therapy.

Please see initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0729.

**Body:****Background:****Oxidative stress and autism:**

Inflammation and systems to combat oxidative stress have been repeatedly implicated in autism, including altered enzyme levels, altered metabolite and biomarker levels and genetic risk factors. Several lines of evidence have been reported implicating oxidative stress as being important in autism. Enzyme activity of several key anti-oxidant genes have been reported altered in autism including reduced enzymatic activities of glutathione peroxidase (GPX) (Golse, Debray-Ritzen et al. 1978, Yorbik, Sayal et al. 2002, Sogut, Zoroglu et al. 2003, Frustaci, Neri et al. 2012, Laszlo, Novak et al. 2013), superoxide dismutase (SOD) (Golse, Debray-Ritzen et al. 1978, Yorbik, Sayal et al. 2002, Frustaci, Neri et al. 2012, Laszlo, Novak et al. 2013) and catalase (Zoroglu, Armutcu et al. 2004, Laszlo, Novak et al. 2013). Another study reported significantly reduced levels of SOD and GPX and increased levels of malondialdehyde in subjects below the age of 6 but not above (Meguid, Dardir et al. 2011). Reduced taurine levels as well as carnosine have been reported (Ming, Stein et al. 2012). In addition, elevated nitrite concentrations have been detected in individuals with autism (Zoroglu, Yurekli et al. 2003) along with thiobarbituric acid-reactive substances (t-bars) and xanthine oxidase activity in red cells (Zoroglu, Armutcu et al. 2004). Increased t-bars,  $\text{Na}^+/\text{K}^+$ -ATPase, isoprostanes and the hexanoyl-lysine adduct (HEL) have also been reported. (Ghezzi, Visconti et al. 2013) Increased non-protein bound iron and 4-hydroxynonenal protein adducts (4-HNE PAs) were also reported increased in autism (Pecorelli, Leoncini et al. 2013).

Glutathione (GSH) levels have been shown to be altered in autism. Total GSH was reportedly decreased while plasma levels of GSSG were elevated; the tGSH:GSSG ratio was low (James, Cutler et al. 2004). A meta-analysis of oxidative stress related biomarkers and genes showed associations with autism for levels of methionine, cysteine, and GSH, glutathione peroxidase but not homocysteine, SOD and

cystathionine (Frustaci, Neri et al. 2012). Significantly reduced activity of key GSH metabolism enzymes was reported in cerebellum of individuals with autism including glutamate-cysteine ligase, modifier subunit (GCLM), GPX and glutathione transferases (GSTs) (Gu, Chauhan et al. 2013), as well as paraoxanase 1 (*PON1*), an enzyme important for hydrolysis of many molecules including organophosphates that is inactivated by oxidized GSH. (Pasca, Dronca et al. 2010)

Several genes relevant to oxidative stress and reducing environmental burdens of oxidative stress have been reported as risk factors for autism including glutathione S-transferase M1 (*GSTM1*), glutathione S-transferase M1 (*GSTP1*), *glutathione peroxidase 1* (*GPX1*), *glutaredoxin 3* (*GLRX3*), *cystathionine gamma-lyase* (*CTH*), *PON1* and glyoxalase I (*GLO1*) (Junaid, Kowal et al. 2004, D'Amelio, Ricci et al. 2005, Buyske, Williams et al. 2006, James, Melnyk et al. 2006, Williams, Mars et al. 2007, Ming, Johnson et al. 2009, Bowers, Li et al. 2011). Rare variants were reported in the 5' region of *SOD1* in a few cases of autism (Kovac, Macedoni Luksic et al. 2014). Last, increases of mitochondrial DNA damage have been reported in autism and this is likely due to an increase in oxidative stress (Napoli, Wong et al. 2013).

#### **Altered lipid metabolism and autism:**

We previously reported increased isoprostanes, auto-oxidation products of the omega-6 lipid arachidonic acid (AA), in autism (Ming, Stein et al. 2005). This was confirmed by others (Yao, Walsh et al. 2006). A third paper confirmed these findings and also reported a more significant increase in F2t-Isoprostanes (F2-IsoPs) levels in autism subjects when stratifying for gastrointestinal problems (Gorrindo, Lane et al. 2013). Another report saw increased 8-isoprostanes, prostaglandin E2 (PGE2) and leukotrienes in 18 Saudi Arabian subjects with autism compared with 20 controls (El-Ansary and Al-Ayadhi 2012). An increase in the omega-6:omega-3 ratio was reported in autism along with increased isoprostanes. Increase of total mono-unsaturated fatty acids (MUFAs) and specifically oleic, palmitoleic and vaccenic acids were reported. Also reported was an inverse correlation with polyunsaturated fatty acids (PUFA) concentration and hyperactivity, an inverse correlation with AA and cognitive impairment and a direct correlation with isoprostane, palmitic acid and total saturated fatty acids. (Ghezzi, Visconti et al. 2013). Levels of omega-6 (AA) and omega-3 (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were reported altered in autism compared to controls. (Bell, MacKinlay et al. 2004) A subsequent study reported an increased AA:EPA (omega-6:omega-3) ratio in autism. (Bell, Miller et al. 2010) It is interesting to note that omega-3 fatty acids in part regulate enzymes of GSH synthesis and metabolism (Arab, Rossary et al. 2006). GSH is important for the synthesis of prostaglandins, leukotrienes as well as for the function of several docosahexaenoic acid (DHA) and AA metabolizing proteins including prostaglandin-endoperoxide synthase 2 (PTGS2, or COX-2) (Chen, Berry et al. 2003, Tsikas, Suchy et al. 2012).

#### **Omega-3 and omega-6 fatty acids:**

Much attention has been paid to the possible role of omega-3 and omega-6 fatty acids in developmental disorders including autism. Omega-3 and omega-6 fatty acids are

particularly important for brain development. They appear to play a role in neuronal maturation and synapse development (Chalon 2006, Cao, Kevala et al. 2009, Dagai, Peri-Naor et al. 2009). Omega-3 fatty acid supplementation has been correlated with positive changes in cognition and behavior in children. (Kuratko, Barrett et al. 2013)

Omega-3 and omega-6 fatty acids are important components of cellular membranes, and their metabolites are important modulators of immune function, inflammation and oxidative stress. They are important parts of several second messenger systems including protein kinase C (Speizer, Watson et al. 1991), cAMP response element binding protein (CREB) and brain derived neurotrophic factor. (Mirnikjoo, Brown et al. 2001, Seung Kim, Weeber et al. 2001). Some preliminary evidence suggests a correlation between plasma fatty acids, particularly DHA and EPA with levels of catecholamine (serotonin and dopamine) metabolites (Hibbeln, Linnoila et al. 1998) and glutamate and serotonin (Chalon 2006, Sharma, Ying et al. 2010) as well as substrates for pro- and anti-inflammatory molecules. Both affect transcription and are important modulators of cytokine levels (McNamara, Jandacek et al. 2010). Enzymes that release DHA and AA from the membrane and metabolize them such as phospholipases and prostaglandin-endoperoxides have been localized to the post synaptic region, an area that has received much attention in autism.

The most abundant omega-3 and omega-6 fatty acids in the brain are docosahexaenoic acid (DHA) with its highest concentration in synaptic membranes and arachidonic acid (AA), respectively. Metabolism of AA, EPA and DHA are in part regulated by neurotransmitters such as serotonin and glutamate, both implicated in autism; serotonin receptor activation results in DHA release from the cell membrane (Rosa and Rapoport 2009). Both DHA and AA modulate NMDA receptor function (Rosa and Rapoport 2009). Chronic NMDA administration in rats leads to increased AA release from the membrane and significantly increased activity of calcium dependence PLA2 (cPLA2), but not calcium independent PLA2 (iPLA2) (Rao, Ertley et al. 2007), which could lead to glutamate excitotoxicity. Omega-3 fatty acid deprivation increases 5-HT turnover as well as pro-inflammatory cytokine levels in rats (McNamara, Jandacek et al. 2010). Increased or deficient levels of DHA and DHA metabolites have been reported to be modulators of glutamatergic synapse function, glutamate transport, GABA receptor-mediated response, serotonin and dopamine pooling and distribution in models systems (Tanaka, Farooqui et al. 2012). Thus, metabolism of DHA and AA is linked to metabolism of glutamate and serotonin.

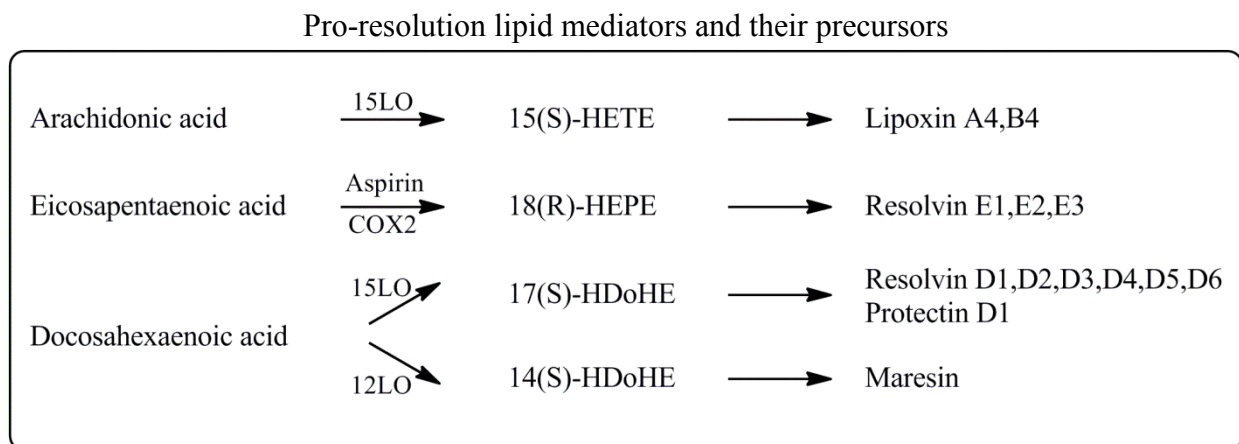
### **DHA and AA metabolites:**

The body's response to infection or injury contains an immediate pro-inflammatory response followed by what is called resolution of inflammation (Fullerton, O'Brien et al. 2014, Spite, Claria et al. 2014). Metabolites of the omega-6 fatty acid AA may be either pro-inflammatory or resolving mediators. These include leukotrienes, prostaglandins and lipoxins. Metabolites of the omega-3 fatty acid DHA are the resolving mediators protectins, maresins and the D class resolvins. Metabolites of the omega-3 fatty acid EPA are the E class resolvins. (Fullerton, O'Brien et al. 2014, Spite, Claria et al. 2014)

A considerable amount of data exists on their normal function in infection and injury. More recently much attention is being given to disorders that have a chronic inflammatory component such as heart disease, asthma and autism.

AA and DHA/EPA are metabolized in related pathways. Membrane-bound AA and membrane-bound DHA are subject to autooxidation to membrane-bound isoprostanes and membrane-bound neuroprostanes respectively. All four compounds are metabolized further by phospholipases A2 (PLA2): membrane-bound AA and isoprostanes by calcium-dependent PLAs and membrane-bound DHA and neuroprostanes by calcium-independent PLAs. The resulting free isoprostanes and free neuroprostanes may appear in blood and the urine. The resulting free AA and free DHA are subject to further metabolism. Lipoxins are derived from arachidonic acid, an omega-6 fatty acid. An analogous class, the resolvins, is derived from DHA and EPA, omega-3 fatty acids. Their appearance in inflammation signals the resolution of inflammation. Free AA is metabolized to lipoxins by ALOX15 (15LO), ALOX12 (12LO), ALOX5 (5LO). Lipoxins are a series of anti-inflammatory mediators. Another class of products of AA metabolism are leukotrienes which require the action of ALOX5 and arachidonate 5- lipoxygenase-activating protein (ALOX5AP). A third class of AA metabolites are the prostaglandins which require action by PTGS1 and PTGS2 (COX 1,2). Free DHA/EPA is metabolized to D and E class resolvins respectively (neuroprotectins) by ALOX15, ALOX12, ALOX5 and by PTGS1 and PTGS2 (COX 1,2). ALOX5 require ALOX5AP. Specific intermediates are listed in Figure #1. Interestingly in addition to the action of its metabolites DHA may be active by itself. It is reported that DHA reduces Cox-2 induction irrespective of its metabolites (Li, Yu et al. 2013).

Figure #1



A very tightly controlled balance needs to exist for normal fighting of infection, repair and resolution of inflammation. A perturbation in this balance has the potential to affect several important systems in autism including inflammation, immune and neurotransmission. Interestingly in a model system omega-3 fatty acid deprivation has been shown to alter the regulation of both DHA and AA metabolizing enzymes. Omega-3 fatty acid deprivation causes a decrease in protein and mRNA levels of the DHA-



metabolizing enzymes iPLA2 and PTGS1. At the same time omega-3 fatty acid deprivation causes an increase in protein and mRNA levels of the AA metabolizing enzymes cPLA2 and PTGS2 (Rao, Ertley et al. 2007) (Kim, Rao et al. 2011). Conversely omega-6 fatty acid deprivation causes an increase in protein and mRNA levels of the DHA metabolizing enzymes iPLA2 and arachidonate 15-lipoxygenase (ALOX15 or 15-LOX). At the same time omega-3 fatty acid deprivation causes a decrease in protein and mRNA levels of the AA metabolizing enzymes cPLA2 and PTGS2 (Kim, Rao et al. 2011). Thus a perturbation of the balance of DHA and AA and their metabolites could affect neurotransmitter function as well as affect the balance of pro- and anti-inflammatory metabolites in individuals with autism.

#### **Other omega-3 and omega-6 treatment in autism:**

Several clinical trials of omega-3 and omega-6 fatty acids have been done in autism. Three used both DHA and EPA. One double blind study reported a non-statistically significant improvement in hyperactivity and stereotypy (Amminger, Berger et al. 2007). a second double blind study reported a non-statistically significant improvement in hyperactivity (Bent, Bertoglio et al. 2011). A non-randomized study with both DHA and EPA reported no improvement (Politi, Cena et al. 2008). Johnson et.al. published a case report of an individual with autism having a reduction in agitation and anxiety when treated with EPA (Johnson and Hollander 2003). A double blind study with DHA and AA was conducted and reported a non-statistically significant improvement in social interaction and a significant increase in plasma levels of superoxide dismutase (Yui, Koshiba et al. 2012). A double blind study using DHA reported no improvement (Voigt, Mellon et al. 2013). Our study is also a double blind study with DHA. Unlike the previous examples of completed studies in addition to possible changes in phenotype we measured changes in urinary biomarkers and genotyped variants in DHA and AA metabolism.

#### **Evidence implicating AA and DHA metabolizing genes in autism:**

Activity of type IV PLA2 was reportedly altered in autism and this change was dependent on omega-3 fatty acid levels (Bell, MacKinlay et al. 2004). Also, *PTGS2* is reportedly associated with autism in a Korean dataset (Yoo, Cho et al. 2008). A study of expression was done in monozygotic twins with different degrees of autism in lymphoblastoid lines and found *ALOX5AP* significantly unregulated in the twin with the more severe phenotype. In addition while not significant, *PTGS2* showed altered expression in at least one twin set (Hu, Frank et al. 2006).

#### **Rationale for AA and DHA/EPA metabolizing genes:**

The pathways for AA and DHA/EPA metabolism are closely related. In addition the pathways of membrane cleavage of these lipids for metabolic use including production of lipoxins and resolvins and that of cleavage of auto-oxidative products (isoprostanes and D and E class neuroprostanes respectively) are similar. In this study we chose 12 genes of DHA and AA metabolism. The calcium-independent phospholipases, **PLA2G6** and, **PLA2G4C** are necessary to release DHA from cell membranes (Rao, Ertley et al. 2007). This is the first step in synthesis of resolvins. **PLA2G4A** and **PLA2G4B** are

cytosolic enzymes important for release of AA and isoprostanes from the membrane. Interestingly a rare mutation in *PLA2G4B* was reported in an individual with autism and suggested to be causal (Matsunami, Hensel et al. 2014). **PLA2G2D** is a secretory-type phospholipase A2-IIID, important for the inflammatory response. **PLAA** is phospholipase A2-activating protein (PLAA), potentially important in regulating the inflammatory response through its activation of phospholipase A2. The lipoxygenases (LO) **ALOX15**, **ALOX12** and **ALOX5** along with its activating factor **ALOX5AP (FLAP)**, are necessary for the production of the anti-inflammatory molecules 16,17-epoxyDHA and resolvins as well as the production of the anti-inflammatory molecule lipoxin and the pro-inflammatory leukotrienes (Bazan 2005). Cyclo-oxygenases **PTGS1 (COX1)** and **PTGS2 (COX2)** are key enzymes in the production of prostaglandins and act as an alternate route of production of resolvins from 16,17-hydroperoxyDHA (Bazan 2005).

### **SOW History:**

This project was designed in the years 2006-2007. The original Statement of Work (SOW) was written at this time. As this project evolved responsibilities were changed. The SOW was amended several times to reflect this. It was amended in March of 2010, July of 2011 and in August of 2012. For all versions of the SOW Day 1 is defined as the award date, 8-16-2008.

We received an IRB approval in December 2009. The changes in the amendment to the SOW of March 2010 are based on the delayed IRB approval. The significant changes in this amendment included a change in timeline and changes in personnel. The timeline for each task was adjusted due to delays in IRB approval. Personnel changes were made as well. Additionally after the departure of the study coordinator, A. Kutlik, Mr. Stenroos from initiating project W81XWH-08-1-0728 wrote an IND letter to the FDA and assisted Dr. Novotny to expedite IRB approval (not reflected in the section of the SOW for W81XWH-08-1-0728).

The amendment to the SOW of July 2011 are also based on the delayed IRB approval as well as to slower recruitment rates than expected. A second change in timeline coinciding with a no cost extension including personnel changes such as adding Dr. Ming to increase subject recruitment, treatment and evaluation was done. In addition responsibilities were added to Mr. Stenroos from initiating project W81XWH-08-1-0728 including consenting of subjects and management of IRB amendments and reviews.

The amendment to the SOW of August 2012 reflects a third change in timeline coinciding with a second no cost extension.

### **Other Important notes:**

Please note. During this project the University of Medicine and Dentistry of New Jersey – Robert Wood Johnson Medical School (UMDNJ-RWJMS) merged with Rutgers University to create Rutgers-RWJMS. In addition, partnering project W81XWH-08-1-0729, originally at UMDNJ-School of Osteopathic Medicine (SOM) is now at Rowan-

SOM. UMDNJ no longer exists but is used in this document up until the time of the merger of RWJMS with Rutgers and SOM with Rowan University on July 1<sup>st</sup> 2012.

Also please note that the company we received the study medication from was called Martek and is currently called DSM. To avoid confusion we use Martek in this document since that was the company name during the project.

**The following people were funded by this project.**

Sherie Novotny, MD.

Annie Kutlik

Shantel Savage

Rakhee Wasiulla

Steven Buyske

Sue Ming

Susan Adubato

Edward S Stenroos

William Johnson

**Project 1: PI Sherie Novotny, MD, Partnering PI, W81XWH-08-1-0730**

Please see initiating project W81XWH-08-1-0728 and partnering project W81XWH-08-1-0729.

[Task #1 Full board review with pending UMDNJ IRB approval as well as obtaining DOD HRPO approval prior to beginning \(Done, S. Novotny\).](#)

**Initial Approval:**

We started the process of applying for an IRB approval once we received notification of funding. A. Kutlik, the study coordinator was responsible for applications, amendments and responses to the IRB. Ms. Kutlik with the help of Mr. Stenroos from initiating project W81XWH-08-1-0728 ,worked with Drs. Novotny, Johnson, and Stein (partnering project W81XWH-08-1-0729) to prepare the IRB application, protocol, consents, assents, supporting documents for IRB review as well as responses to de-briefing memos. Our application was submitted on July 18<sup>th</sup> 2008.

We received a de-briefing memo from the IRB on August 4<sup>th</sup> with several minor requested changes. We finalized and sent the responses to these concerns on September 15<sup>th</sup>, 2008.

A second de-briefing memo was received with an additional question. We responded to this additional question on November 5<sup>th</sup>, 2008. Our IRB office moved locations in October and in the process it appears that some material from our previous response on September 15<sup>th</sup> were lost. We re-sent all material to the IRB on November 21<sup>st</sup>.

We received an "Approval with Stipulations" from our IRB on November 26<sup>th</sup>, 2008. We responded to the four stipulations, and an expedited review was scheduled for January

9<sup>th</sup>. 2009. The reviewer decided that our first response (related to simplification of the language in the consent form) should be reviewed by a full committee. The committee met on January 30<sup>th</sup>, 2009. At this meeting our four responses were tabled and 24 suggestions / recommended changes were sent to us. Many of the requests were minor but some were significant. One of these changes requested was that we obtain an IND from the FDA for use of the study compound DHA.

Our response to this major concern contained copies of documentation to support that an IND was not needed because 1.) an FDA letter dated May 17, 2001 to Martek, manufacturer of the DHA used designated their DHA as "Generally Regarded as Safe" (GRAS) (please see <http://www.cfsan.fda.gov/~rdb/opa-g041.html>) and 2.) documentation from [www.clinicaltrials.gov](http://www.clinicaltrials.gov) DHA) showing that none of the then 10 current or completed studies that used Martek's DHA had needed an IND. This was including one with subjects with autism which showed that our use was not a new indication. We responded to this and the other 23 new questions and submitted for review on February 27<sup>th</sup>, 2009.

We received a de-briefing memo on March 6<sup>th</sup>, 2009. Our IRB did not accept our response and we were told that we would need to get an IND from the FDA for the project. Two 2 new additional significant requirements were included. First, our IRB wanted us to create a tissue bank for the storage of the samples. Second our IRB wanted us to apply for a Certificate of Confidentiality for this project before they would give approval.

We convened a meeting with the Chair of the IRB, the IRB director and the PI's on May 8<sup>th</sup>, 2009 to discuss each of their requirements.

The key outcomes of the meeting were as follows;

First, even though it is already considered General Regarded as Safe (GRAS) in children we would need either an IND for the use of Martek's DHA or a letter from the FDA saying that one was not needed. During this process the study coordinator for the project, A. Kutlik tendered her resignation for her position and later left the University (June 30<sup>th</sup>). Shantel Savage took over as study coordinator with the help of Mr. Stenroos, initiating project W81XWH-08-1-0728. Mr. Stenroos wrote and submitted an IND application. The IND was submitted on July 16<sup>th</sup>, 2009 (available upon request, 298 pages). We received a letter from the FDA August 4<sup>th</sup>, 2009 exempting us from needing an IND.

Second, even though to our knowledge there was no federal rule or regulation requiring this, a tissue bank application, protocol, manual along with supporting documents would have to be written and submitted for this project. This has been done by Dr. Johnson and Mr. Stenroos from initiating project W81XWH-08-1-0728. Initial application was on May 24<sup>th</sup> 2009. For additional information regarding the establishment of the Tissue Bank please see "final report for initiating project W81XWH-08-1-0728".

Third, our IRB requested that we apply for a Certificate of Confidentiality (COC) before they would give us full approval (as opposed to after). IRB approval is a requirement from the NIH for a COC application. In our correspondence with the NIH we were told that a provisional approval contingent on a COC would be acceptable for us to submit an application. Therefore our IRB agreed that when all other matters were satisfied we would receive an approval conditional on obtaining a COC.

Additional submissions, responses and amendments.

The following dates represent requests for changes or requests for clarifications (to either the consent form, assent form, protocol, application or other supporting documentation or recruitment materials) and subsequent replies by us;

|                   |                                   |  |
|-------------------|-----------------------------------|--|
| Memo from the IRB | May 29, 2009                      | Replied to on July 10 <sup>th</sup> , 2009     |
| Memo from the IRB | September 1, 2009                 | Replied to on September 9 <sup>th</sup> , 2009 |
| Memo from the IRB | September 30 <sup>th</sup> , 2009 | Replied to on October 19, 2009                 |
| Memo from the IRB | November 20 <sup>th</sup> , 2009  | Replied to on December 5 <sup>th</sup> , 2009  |

We obtained IRB approval on December 7<sup>th</sup> 2009. Two important points: First our IRB office accepted the FDA's letter stating that we do not need an IND. Second, our IRB office accepted a proposal to allow the protocol to include the destruction of samples at the end of the project (with the understanding that once a tissue bank was created an amendment would be submitted to re-consent the subjects and entered them into the newly established tissue bank).

We submitted our approval and all relevant documents to the Human Research Protection Office (HRPO), Office of Research Protections (ORP) of the DOD for review on December 10<sup>th</sup> 2009. We received requested changes from the office on January 9<sup>th</sup> 2010

We applied for a COC December 15<sup>th</sup>, 2009 and received it on March 2<sup>nd</sup> 2010. The COC and the requested changes from the DOD Human Research Protection Office (HRPO), Office of Research Protections (ORP) review were submitted as an amendment to our IRB office on March 8<sup>th</sup>. This went for an expedited review and we were informed on March 25<sup>th</sup> that the amendment was accepted. All relevant documents were sent to the Human Research Protection Office (HRPO), Office of Research Protections (ORP) of the DOD on March 30<sup>th</sup> 2010.

#### **DSMB:**

We organized the creation, interviewed and invited members to participate in our Data and Safety Monitoring Board (DSMB). The members of our DSMB was finalized in September of 2010. The initial members were Dr's Wei-Ting Hwang of University of Pennsylvania, Kapila Seshadri of UMDNJ-RWJ and Bart Kamen of UMDNJ-RWJMS. The Data Safety Monitoring Board (DSMB) received a copy of the most recent continuing review (CR) and all updated materials. They held their initial meeting and we received approval to start recruiting on November 12<sup>th</sup> 2010.

**The first annual DSMB review:**

Mr. Stenroos supplied the board with the current approved continuing review and related documents along with our report on October 21<sup>st</sup> 2011. The breach of protocol mentioned (see section “**Breach of Protocol**”) was also reported. The first annual review occurred on November of 2011. We were notified on November 18<sup>th</sup> 2011 of their approval to continue the study.

**The second annual DSMB review:**

We began the process of preparing for the second annual DSMB review when we learned that one of the members, Dr. Kamen died. Mr. Stenroos requested and received instructions on how to replace a member of the board and did so. Dr. Patricia Sonsalla UMDNJ-RWJMS replaced the late Dr. Kamen. Once this was done Mr. Stenroos sent the board the current Continuing Review and supporting documents along with our report to the DSMB and the report was approved on 2/13/2013 and we received a letter from the board members on 3/13/2013.

**The third annual DSMB review:**

We sent our current protocol and CR with supporting documents along with our report to the DSMB in early May 2014. Two requests for clarification were made by a member of the board. These were answered and the report was approved on 5/20/2014.

**Martek (later named DSM):**

A requirement of receiving the study material was an approved IRB protocol. Mr. Stenroos from initiating project W81XWH-08-1-0728 submitted the fully approved protocol to the supplier of the DHA and Placebo (Martek) along with all relevant documents and medical licenses and a completed FDA form 1572 on April 1<sup>st</sup> 2010 as required to initiate a materials transfer agreement. On April 15<sup>th</sup> 2010 we received an e-mail from a Martek representative. They requested that we change our IRB protocol by significantly increasing the dosage to “maximize the chances of success”. We strongly disagreed with their suggestion and prepared and sent a response. The response was based on studies showing that too large a dose could change normal processing of DHA as well as excretion of biomarkers. A tele-meeting was held between a representative from Martek and Mr. Stenroos on April 23d 2010. It was then agreed that our protocol was correct and acceptable. Martek then went to work on the material transfer agreement. We received the agreement on May 19<sup>th</sup> 2010. Upon review there were two items of concern. 1.) Martek was asking for all patent rights on the project. 2.) Martek was asking the University and therefore the State of NJ to take responsibility for insurance costs if there was an adverse effect. Naturally, neither of these items is acceptable to the University or the State of NJ. We had a meeting with our lawyer responsible for negotiating this agreement on May 27<sup>th</sup> 2010 and a follow up meeting to finalize the response on June 9<sup>th</sup> 2010. Our licensing department sent a reply on June 17<sup>th</sup> 2010. The response to the first item was to notify Martek what patents had previously been applied for, therefore indicating the University's ownership of Intellectual Property related to the project. In addition our licensing department suggested that all intellectual property solely developed by Martek should be patented

by Martek, All intellectual property solely developed by us should be patented by us and all intellectual property developed together should be shared equally. The response to the second item was to notify Martek as to what is legal and acceptable to UMDNJ and the State of NJ. We received a response from Martek on July 20<sup>th</sup> 2010. We received the final Materials Transfer agreement from Martek on August 4<sup>th</sup> 2010. We returned the signed copy to Martek on August 9<sup>th</sup> 2010. We received the DHA and Placebo August 20<sup>th</sup> 2010.

Mr. Stenroos acted as liason with Martek in all matters including receipt of original materials and receipt of replacement materials to ensure freshness in the fall of 2013.

#### **Pharmacist:**

Upon scheduling our first subject to start DHA/Placebo treatment we were informed that the pharmacist who is to distribute the DHA and Placebo and the administrator in charge wanted to re-review the protocol and the study. We supplied them with the current protocol and all supporting documents for review in February 2011. They agreed to dispense the DHA/Placebo in April 2011.

#### **Continuing Review (CR):**

The Initial IRB approval issued December 7<sup>th</sup> 2009 had an expiration date of October 1<sup>st</sup> 2010. Therefore our first CR application was prepared and submitted with no changes to the protocol, consent or supporting material to our IRB. We received a de-briefing memo with five concerns. We responded to this and we were issued an approval pending 4 minor changes on October 4<sup>th</sup> 2010. We made the recommended changes and submitted to our IRB. We received a CR November 10<sup>th</sup> 2010. The approved CR was sent to the HRPO on November 11<sup>th</sup> 2010. Additional clarifications were requested by and provided to the HRPO over the following few weeks.

We submitted our second CR application to our IRB on August 26<sup>th</sup> 2011. Upon review of our CR application our IRB sent us a de-briefing memo on September 13, 2011 which made several requests/changes mostly to reword parts of the application, protocol and consent. Along with this was the addition of "Genetic Information Nondiscrimination Act (GINA)" text. We submitted these changes on September 14<sup>th</sup> 2011. We received another de-briefing memo on September 27, 2011 with additional requested changes in text in the protocol and application. We submitted our responses on the same day. We received approval on September 29<sup>th</sup> 2011. The approved CR was sent to the HRPO on October 1<sup>st</sup> 2011.

We submitted our third CR application to our IRB on August 22<sup>nd</sup> 2012. We received a debriefing memo on September 10, 2012 containing 7 changes to the application, protocol, consent and assent form. We submitted our responses on September 13<sup>th</sup> 2012. We received an approval on September 27<sup>th</sup> 2012. The approved CR was sent to the Department of Defense (DOD) Human Research Protections Office (HRPO) on October 4<sup>th</sup> 2012.

Our fourth CR was submitted on August 13<sup>th</sup> 2013 and was approved on October 7<sup>th</sup> 2013. While this project is closed, partnering project W81XWH-08-1-0729 was given an additional-cost extension to recruit additional subjects so the project protocol has remained open for recruitment. Additionally, since the Rowan-SOM IRB office relevant to Dr. Stein's project was part of the UMDNJ-IRB until July 1<sup>st</sup> 2013 it was decided by both IRBs that a review and an approval by the Rutgers-RWJMS IRB would be sufficient for an approval for the Rowan-SOM IRB.

**Ammendments:**

Before the initial IRB approval we submitted 3 modifications to the protocol. All related to changes in study personnel between the time the grant was funded and the time the study received IRB approval. This includes the addition of S. Savage as an assistant study coordinator in January of 2009, the removal of A. Kutlik the study coordinator and S. Savage the assistant coordinator in July 2009 and the addition of R. Wasiulla as study coordinator in September 2009. Mr. Stenroos assisted Dr. Novotny in between the removal of S. Savage and A. Kutlick and the addition of R. Wasiulla in the end of September.

After our initial IRB approval we submitted the COC and the requested changes received from the Human Research Protection Office (HRPO), Office of Research Protections (ORP) of the DOD on January 9<sup>th</sup> 2010 were submitted as an amendment to our IRB office on March 8<sup>th</sup>. This went for an expedited review and it was accepted on March 25<sup>th</sup> 2010. The approved modification and all relevant documents were sent to the Human Research Protection Office (HRPO), Office of Research Protections (ORP) of the DOD on March 30<sup>th</sup> 2010.

As per the request of the Human Research Protection Office (HRPO), Office of Research Protections (ORP) of the DOD we submitted an amendment to our IRB on July 6<sup>th</sup> 2010 to change the backup for Dr. Novotny from Dr. Petti to Dr. Lambert to avoid a potential conflict of interest. The amendment was approved on July 22<sup>nd</sup> 2010.

We submitted an amendment to our IRB to add additional sources of subjects along with our first CR submission. These include fliers for posting, a webpage and a weblink. We received approval on December 9<sup>th</sup> 2010. The webpage and webpage link were submitted to the major patient advocacy/support group in NJ called Autism NJ for review. The material was approved and the recruitment flier link was posted on their website on December 14<sup>th</sup>, 2010.

A second amendment to add two additional sources of subjects was submitted on December 10<sup>th</sup> 2010. The first was to allow subjects of past studies that expressed an interest in being part of future studies to be contacted. The past studies include a study done by Drs. Johnson and Mars, a previous study by Dr. Ming and a previous study done by Dr. Lambert. An advantage to using these subjects is that they have already undergone ADOS and ADI. The second source was the Douglass Developmental Disabilities Center (DDDC) of Rutgers University allowing a letter to be sent to DDDC



families that previously expressed an interest in being part of research projects. We received IRB approval to distribute the recruitment letters on February 4<sup>th</sup> 2011.

We submitted an amendment to our IRB containing a number of issues including modifying recruitment tools and two changes to the protocol removing two blood tests on May 9, 2011. The amendment contained two types of changes. Recruitment and removal of two tests deemed no longer useful to the project and therefore an unnecessary extra burden on the subjects and their families. We re-wrote our flier and made additional fliers targeted to different audiences (doctors of potential autism subjects, families etc). Two blood tests were removed from the protocol. In brief, to decrease the burden on the subjects we removed the PT/PPT bleeding profile. It is known that there is a risk of reduced platelet activity and increased bleeding times when very large doses of omega-3 fatty acids are given. We learned that decreased platelet function and increased bleeding times due to high doses of fish oil are due to the action of eicosapentaenoic acid's (EPA's) down regulation of *PGE2* and *TXA2* (Bernardini, Chiarenza et al. 1989). EPA is a different component of fish oil and the tablets we were using contain no EPA. In addition Nelson, GL et al 1997 (Nelson, Schmidt et al. 1997) previously tested DHA to see if there was any effect of DHA on bleeding time. They gave 15 grams (375 times the dosage we are using) of DHA per day for 90 days and saw no changes in platelet aggregation or prothrombin bleeding time. We therefore felt that the increased burden on the subjects of requiring additional blood drawing was not productive and did not increase the safety of this trial. In addition, in our original study design when the grant was written in 2006-2007 we had planned on testing certain environmental toxins. As results from other studies became available including Dr. Lambert's study we decided that the tests would not be scientifically productive and we decided it would not be proper to require the subjects to give additional blood for these tests. We therefore removed them from the study and the protocol. A clerical error on our part reintroduced the text back into the protocol and this text was in the final IRB approved version. This amendment was approved on August 2<sup>nd</sup> 2011. Four subjects were enrolled during the review process and we did not draw blood for these two tests which represents a protocol deviation (please see section "Breach of Protocol").

An amendment was submitted to our IRB with our second CR on August 26<sup>th</sup> 2011 to restructure partnering project W81XWH-08-1-0730 (Novotny) for recruitment of subjects along with a re assignment of tasks. In brief, we identified two areas that appeared to restrict recruitment. First, we found that families that did not live close to the Piscataway/New Brunswick campus where we were recruiting, consenting, randomizing and testing subjects were less likely to enroll. Second, as designed recruiting, initial evaluation, randomization, consenting and testing are all in the hands of either Dr. Novotny or Ms. Wasiulla. We addressed both issues at the same time. First, we amended the protocol to allow Dr. Ming to recruit her patients, do preliminary screening, consenting and test subjects. In this new model we divided recruitment into two areas. Dr. Novotny was responsible for recruitment in the New Brunswick/Piscataway area (central NJ) and Dr. Ming was responsible for recruiting in the Newark area (northern central NJ). Our study coordinator Ms. Wasiulla split her time between the New Brunswick / Piscataway area and Newark. This way all parts of the trial were done

where the subject was recruited (with the exception of the randomization). This amendment was approved with the CR on September 29<sup>th</sup> 2011.

We submitted an amendment on January 18<sup>th</sup> 2012 to allow us to recruit from AutismMatch. Included in this amendment were a new consent, a new flier and an updated protocol. This amendment was approved on February 29<sup>th</sup> 2012.

Our study coordinator, Rakhee Wasiulla tendered her letter of resignation in January 2012 and she left the project in mid February 2012. Her responsibilities were taken over by Dr's Novotny, Ming and Adubato and Mr. Stenroos from initiating project W81XWH-08-1-0728. Mr. Stenroos took over all efforts of recruitment. An IRB amendment was submitted to update recruitment materials. All references to Dr. Wasiulla and her contact information were changed to Dr. Novotny and her contact information.

An amendment was submitted to add Karan Grover to the study to help Dr. Ming with subjects on August 15<sup>th</sup> 2013. It was approved on October 7<sup>th</sup> 2013

#### **Breach of Protocol:**

In our original study design, 2007 we had planned on testing certain environmental toxins. As results from other studies became available including Dr. Lambert's study we decided that the tests would not be scientifically productive and we decided it would not be proper to require the subjects to give additional blood for these tests. We therefore removed them from the study and the protocol. A clerical error on our part reintroduced the text back into the protocol prior to initial IRB approval. We noticed this and submitted an amendment to fix the issue. The amendment was approved on August 2<sup>nd</sup> 2011. Four subjects were enrolled prior to this amendment. Blood samples were not drawn for these previously removed tests which represents a protocol deviation. We informed our IRB and the DOD of this. We received a letter accepting our report and determined that the subjects were not exposed to any additional risks from our IRB on August 2<sup>nd</sup> 2011. A copy was sent to the DOD HSROP on August 15<sup>th</sup> 2011. This information and the IRB letter was also supplied to our DSMB.

#### **GINA letters:**

As per our IRB we began sending Genetic Information Nondiscrimination Act (GINA) letters to our subjects that were consented before our last Continuing Review when GINA language was added.

**Task 2 Volunteers recruited from local clinics, support groups and advocacy groups (6-30 months).Forty four child or adolescent outpatients per year with age ranges from 5-17, for three years totaling 132 patients, will be randomized into the 12-week double-blind, placebo-controlled parallel treatment study.**

Lists of subjects interested in participating in studies were organized by contacting special schools, pediatricians, psychiatrists and special educators in the Middlesex County, where we are based, as well as the neighboring counties. Letters to these

professionals have helped in bringing forward many potential subjects who have been contacting Dr. Novotny.

Recruitment letters were written and distributed for several sources. The DDDC, for Drs. Ming, Johnson and Lambert to send to their subjects from previous studies that wished to be contacted about future studies. In addition a "Dear Dr. letter" was written. A web page was set up and posters generated for posting at local autism centers.

The trial was registered at Clinical trials.gov by Rakhee Wasiula in 2010.

Throughout the study we continually updated and improved our recruitment techniques and tools

Due in part to reduced recruitment due to travel issues we restructured our recruitment model. In this new model we divided recruitment into two areas with Dr. Novotny responsible for recruitment in the New Brunswick/Piscataway area (central NJ) and Dr. Ming is responsible for recruiting in the Newark area (northern central NJ). Our study coordinator Ms. Wasiulla split her time between the New Brunswick / Piscataway area and Newark to complete psychological testing for diagnostic confirmation and other baseline behavioral testing.

We also contacted the director of Children's Specialized Hospital which has several branches in NJ. These hospitals cater to a large section of children and adolescents with mental health issues in NJ. We met with Dr. Mintz of The Center for Neurological and Neurodevelopmental Health in 2011.

Dr. Ming contacted others at the Children's Specialized Hospital at Mountainside. Dr. Evelyn Okouneva was a graduate of Dr. Ming's Pediatric Neurology program who works at Children's Specialized Hospital at Mountainside, NJ. St. Peter's Hospital. Dr. Barbie Zimmerman-Bier runs an autism center at St. Peter's hospital and sees patients 2 days a week.

We attended the annual conference of Autism NJ in 2011. This conference is the largest of its kind in the state. It is a large and valuable tool and is attended by families of individuals with autism, doctors, representatives of autism schools and autism centers/departments of hospitals as well as researchers. We have attended before and it has been a valuable source in the past. Unfortunately we were unable to utilize fully last year since we did not have full approval at that time.

Dr. Novotny will make a presentation titled "Alternative Treatments in Autism" at the conference. In her talk she will be speaking of the use of Omega-3 fatty acids in autism.

We had a booth to distribute our recruitment materials and Dr's. Novotny, Ming and Wasiulla and Mr. Stenroos attended the conference with the goal of informing autism families, doctors, representatives of autism schools and autism centers/departments of hospitals as well as researchers of our study and making contact with potential recruitment sources.

We added AutismMatch as a source of recruiting (please see Task1).

**Task 3 Informed consent/assent obtained (6-51 months).**

This task has been completed. We obtained consent/assent from 49 subjects and assented 2.

**Task 4 Full diagnostic assessment with Autism Diagnostic Interview-revised (ADI-R), Autism Diagnostic Observation Scale (ADOS), Vineland Adaptive behavior scale and Leiter Intelligence Scale (E Roberts); DSM IV criteria (S. Novotny) for eligibility & diagnosis. Parents will complete baseline Aberrant Behavior Checklist; study psychiatrist will complete Clinical Global Improvement, baseline Severity Scale (6-30 months, 21-51 months in the current SOW).**

This task has been completed. Fifty one subjects have undergone ADOS, ADI, Vineland and Leiter.

**Task 5 Cases undergo full medical evaluation to determine health; at this visit will have phlebotomy including 10 mls for blood chemistry, PT/PTT, hematology, 10 ml for genotyping (Project III), urine for pregnancy test, drug screen as indicated, routine urinalysis; urine collected for Project II.**

This task has been completed. 44 subjects underwent medical examination. One subject was removed after initial blood chemistry and lab tests.

**Task 6 Cases randomized to receive either DHA, 200mg daily, or placebo. Cases given DHA after physical exam and routine lab-work completed.**

This task has been completed. 43 subjects were randomized and received either DHA, 200mg daily, or placebo, 11 of which dropped out.

**Task 7 Cases seen weekly for four weeks and biweekly for the remaining 8 weeks. Aberrant Behavior Checklist done every 4 weeks and at termination and Clinical Global Improvement Scale done every 2 weeks and at termination.**

This task has been completed. Thirty two subjects have completed each of the study scales and checklists at each time point.

**Task 8 Cases complete the study with repeat ADOS, Vineland Adaptive Behavior Scale (E Roberts) and Aberrant Behavior checklist (parent) and Clinical Global Improvement Scale (S Novotny). Blood work for safety measures; urine will be collected for Project II during last week of DHA or placebo.**

This task has been completed. Thirty two subjects have completed the study and therefore this task.

**Task 9 Data will be collected and analyzed (6-36 months, 04 year as per the current SOW S Buyske).**

An interactive database for compilation of phenotype data was built and data entered in preparation for analysis. We are still in the process of data analysis.

**Task 10 Manuscripts prepared and submitted for publication (03 year, 04 year as per the current SOW all investigators)**

This task is to be done when the analysis of the data is completed.

**Key Research Accomplishments**

None at this time.

**Reportable Outcomes:**

Dr. Novotny gave a presentation titled “Alternative Treatments in Autism” at the Autism NJ conference in 2011. She spoke of the use of Omega-3 fatty acids in autism.

Dr. Novotny gave a Grand Rounds in Jersey Shore Medical center, February 2013 with the title “Alternative Treatments in Autism”.

**Conclusion:**

This project was unexpectedly and significantly delayed for a number of reasons including several unforeseen additional requirements to receive an IRB approval. The most significant was the requirement that an IND application be submitted to the FDA. This requirement was made 6 months after our initial IRB application was submitted. Our response to this major concern contained copies of documentation to support that an IND was not needed because 1.) an FDA letter dated May 17, 2001 to Martek (manufacturer of the DHA to be used) designated their DHA as “Generally Regarded as Safe” (GRAS) (please see <http://www.cfsan.fda.gov/~rdb/opa-g041.html>) and 2.) documentation from the website [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (search for MARTEK and DHA) showing that none of the then 10 current or completed studies that used Martek’s DHA had needed an IND. This included one with subjects with autism which showed that our use was not a new indication. Our IRB did not accept this and so we were forced to apply for an IND. We did so and received an exemption letter from the FDA on August 4th, 2009. We received our initial IRB approval on December 7th 2009, about 17 months after our initial application. A second additional requirement was to apply to NIH for a Certificate of Confidentiality which we did as soon as we received IRB approval. The COC and requested changes made by Human Research Protection Office (HRPO), Office of Research Protections (ORP) of the DOD were submitted to our IRB

as an amendment. This was approved on March 25th, 2010. At this point we had all approvals to begin and started recruiting subjects. In April of 2010, in anticipation of having final approval we began the process of obtaining the study material from DSM, formerly called (Martek). A number of issues delayed implementation of the Materials Transfer agreement which was finally approved by both parties on August 9th 2010 and we received the study materials on August 20th 2010. The study materials were brought to the University Pharmacist at which time he decided to re-review the study protocol. He agreed to dispense study materials in April 2011. Treatment started immediately on recruited subjects.

Subject recruitment was much slower and lower than anticipated due to several reasons. While a large number of inquiries were made about this study from our various recruiting sources. Many families decided to not join the study. First, this project was designed in 2006. By the time we started recruiting subjects many were already taking fish oil or omega-3 supplements. Second, many that were not taking supplements chose not to join the project unless they were guaranteed to be given DHA and not placebo. Their rationale was that if they were not given DHA they could just go to the store and buy for themselves. Last, due to the nature of the time commitment of this project travel turned out to be an issue for many possible subjects. We made adjustments including starting to test and treat subjects in a second location to help alleviate this problem. Lastly, many of our recruiting sources turned out to be not productive. We obtained consent/assent from 49 subjects and assented 2. All of these 51 subjects underwent initial phenotype testing including ADOS, ADI, Vineland and Leiter. Forty four of these subjects underwent medical examination. One subject was removed after initial blood chemistry and lab tests. Forty three subjects were randomized and received either DHA, 200mg daily, or placebo, 11 of which dropped out. Thirty two of these subjects completed each of the study scales and checklists at each time point and completed the study. We are currently in the process of analyzing our data. We will finish analysis as soon as possible. If we are able to find additional funding we will modify and re-open this project to recruit new subjects to increase our statistical power. We plan on following up any positive data with grant applications to extend our findings as well as those of Partnering project W81XWH-08-1-0729 and Initiating project W81XWH-08-1-0728.

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